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# Microheterogeneity of *S*-glycoprotein of mouse hepatitis virus temperature-sensitive mutants

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## Summary

Mouse hepatitis virus (MHV) strain JHM (MHV-JHM) is a neurotropic coronavirus that causes acute fatal encephalomyelitis in 75–99% of infected mice. The surviving animals may subsequently develop demyelinating disease. We compared the *S* peplomer protein of the wild type (wt) and five temperature-sensitive (ts) mutants of MHV-JHM. In contrast with the wt, none of these five cause fatal disease (mortality less than 10%). Three of these ts mutants did not induce any demyelinating disease, a fourth caused demyelinating disease in 5% of the animals and a fifth, designated ts8, exhibited strong demyelinating properties and caused demyelination in 99% of the animals. SDS-PAGE analysis revealed no differences in the molecular weight of *S* peplomer protein of wt or ts MHV-JHM mutants. However, isoelectric focusing of the *S* protein of these five ts mutants and the wt MHV-JHM, followed by transfer to nitrocellulose sheets and immunoblotting with anti-*S* specific antibody revealed significant differences in the microheterogeneity of the *S* protein.

Mouse hepatitis virus

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*Abbreviations:* IEF, isoelectric focusing; NC, nitrocellulose; TBS, Tris-buffered saline pH 8.3; TBS-milk, TBS containing 3% nonfat dry milk and phenyl-methyl sulfonyl fluoride; PBS, phosphate-buffered saline, pH 7.2.

## Introduction

Isoelectric focusing (IEF) is a powerful method that has been employed for isolation and characterization of many structural and non-structural viral proteins. Because of its ability to perform high resolution separations, IEF is able to detect single amino acid substitutions. It has been used for the classification of viruses and strain identification, physical mapping of mutations, studies of precursor product relationships and of genetic recombination among RNA viruses (Trent, 1977; King and Newman, 1980; Donaldson et al., 1982; King et al., 1982; Dawe and King, 1983). IEF of intact virions allowed the differentiation of rabbit from human papilloma viruses, polioviruses from echoviruses and smallpox from whitepox (Zerda and Gerba, 1985). IEF has also been employed in studying the development of charge heterogeneity in individual viral proteins, known as microheterogeneity (Hsu and Kingsbury, 1985; Bosch, 1985). Microheterogeneity is usually the result of post-translational modification, causing the addition of negatively charged groups, such as sialic acid or sulfate in the oligosaccharide site chains of glycoproteins (Hubbard and Ivatt, 1981). Phosphorylation may be the cause of charge heterogeneity of non-glycosylated viral proteins.

Combining of IEF in polyacrylamide gels with identification of the separated proteins using antibodies (Catsimpoolas, 1969), further enhanced the usefulness of IEF for the characterization of proteins. In addition, IEF has been used to separate antibody clonotypes which were subsequently either immobilized in the isoelectric focusing gels or transferred to nitrocellulose (NC) membranes and detected by reacting them with the appropriate antigens or haptens (Askonas et al., 1970; Keck et al., 1973; Nye and Roitt, 1980; Vandvik et al., 1982; Friedenson and Soong, 1984). Knisley and Rodkey (1986) developed a method, designated affinity immunoblotting, for analyzing clonotypic changes of specific antibody produced during the immune response to a particular antigen, by reacting IEF separated antibody molecules with antigen-coated NC membranes. The specific antibody molecules were bound to the antigen on the NC membranes and were detected using peroxidase-conjugated anti-IgG.

The use of isoelectric focusing followed by western blotting is reported for comparison of the *S* peplomer protein of wild type (wt) and temperature sensitive (ts) mutants of the mouse hepatitis virus (MHV) strain JHM (MHV-JHM).

## Materials and Methods

*Cells and Viruses* The origin and growth of the L2 cell line has been previously described (Rothels et al., 1959). Cells were propagated in Dulbecco's Modified Eagle Medium (DMEM, Gibco Laboratories, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS) and a 100 units/ml of

penicillin G and 100 micrograms/ml of streptomycin. wt MHV-JHM employed in these studies has been described previously (Oleszak and Leibowitz, 1990a). Five independent RNA+ ts mutants of MHV-JHM, namely ts104, ts110, ts115 (Robb et al., 1979; Bond et al., 1979), ts8 and ts15 (Haspel et al., 1978), have been employed in this study and their characteristics are summarized in Table 1. Propagation of the ts104, ts110 and ts115 mutants and of wt MHV-JHM was carried out as described (Robb et al., 1979; Bond et al., 1979).

*Antibodies* The 1.38.1 monoclonal antibody (mab) specific for the *S*-glycoprotein of MHV and the 1.16.1 mab specific for the *N*-glycoprotein of MHV have been described elsewhere (Leibowitz et al., 1987; Oleszak and Leibowitz, 1990a). A hyperimmune goat serum specific for the *S*-glycoprotein of MHV was a generous gift from Dr. K. Holmes (Uniformed Services University for Health Sciences, Bethesda, Maryland) and it has been characterized previously (Oleszak and Leibowitz, 1990a). The J2.7 mab specific for the *M* protein of MHV was kindly provided by Dr. Fleming (Univ. of Wisconsin, Madison, WI). Affinity purified goat anti-mouse IgG, heavy and light chain-specific, was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

*Preparation of cell lysates* Monolayers of L2 cells were mock infected, or infected with either wt or ts mutants of MHV-JHM at multiplicity of infection of 0.1. For the immunoprecipitation SDS-PAGE experiments described below cells were labeled 12 h after infection with 300 microCuries/ml of $^{35}S$ in methionine-free DMEM. In contrast, non-labeled lysates were used in the IEF experiments. Cells were lysed 11 h later (ts mutants) or 3 h later (wt MHV-JHM) in lysing buffer comprised of 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40 and 0.2 trypsin-inhibiting units/ml of aprotinin (all purchased from Sigma), as previously described (Oleszak and Leibowitz, 1990a, 1990b).

*Immunoprecipitation and SDS-PAGE* Aliquots of 50 microliters of cell lysates were immunoprecipitated with appropriate antibodies using *Staphylococcus aureus* Cowan strain cells (Calbiochem, San Diego, California) as described elsewhere (Oleszak and Leibowitz, 1990a; Oleszak et al., 1992). Immunoprecipitates were heated at 70°C for 5 min in SDS–PAGE sample buffer and analyzed on 10% SDS-PAGE as previously described (Oleszak and Leibowitz, 1990a; Oleszak et al., 1992).

*Isoelectric focusing* IEF was performed in an LKB Multiphor apparatus (LKB, Piscataway, NJ) thermostatted at 8°C. Polyacrylamide gels (25 × 12 × 0.2 cm) consisted of 5.3% T, 3% C, 3% ampholyte, 8 M urea, and 0.1% NP-40. Ampholytes were synthesized by the method of Binion and Rodkey (1981) from pentaethylene hexamine and acrylic acid. Electrode wicks were saturated with 1 M NaOH (cathode) and 1 M $H_3PO_4$ (anode). 75 microliters of lysate

(non-labeled) prepared as described above, was applied to the gel surface using pieces of Whatman no. 1 filter paper. The IEF protocol began with an initial setting of 100 V (constant voltage) for 30 min, 200 V (constant voltage) for an additional 30 min, then 20 W (constant power) for 30 min, and 30 W (constant power) for an additional 2 h. The pH gradient was measured immediately after focusing using a flat surface pH electrode. The recorded pH values were adjusted according to Gelsema et al. (1979) by subtracting 0.5 from each value to compensate for the effect of 8 M urea on the apparent p*I*.

*Nitrocellulose blotting* NC sheets (0.45 $\mu$m, Schleicher and Schuell) were hydrated in distilled water and subsequently equilibrated in distilled water containing 8 M urea prior to blotting. Following the IEF run, the NC sheet was removed from the urea solution and blotted on filter paper to remove excess fluid. The NC sheet was carefully placed over the focused lanes of lysate and incubated at room temperature for 20 min to allow focused proteins to bind to the NC sheet. After incubation, the NC-antigen sheet was rinsed 3 times with PBS, rocked for 30 min and again rinsed 3 times with phosphate buffered saline, pH 7.2 (PBS). The NC-antigen sheet was blocked overnight at 4°C in Tris-buffered saline, pH 8.3, containing 3% non-fat dry milk and 1 mM phenyl methyl sulfonyl fluoride (TBS-milk). After 18 h this solution was replaced with TBS-milk containing 1:33 diluted goat anti-*S* serum (described above) and again rocked overnight at 4°C. The blot was rinsed four times in TBS-0.05% Tween 20, rocked for 40 min with several changes of TBS-0.05% Tween 20. Bound antibodies were detected by incubating the sheets with horseradish peroxidase-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch Laboratories) diluted 1:500 in TBS-milk for 1 h at 4°C. The blot was rinsed four times with PBS-0.05% Tween 20, rocked for 40 min with several changes of PBS-Tween and finally rinsed with PBS. Bound antibodies were visualized by immersing the blot in substrate solution (0.6 mg/ml diaminobenzidine, 0.03% hydrogen peroxide in PBS) for 5 min at room temperature. After band development, sheets were rinsed thoroughly with water and air dried.

## Results and Discussion

The characteristics of the MHV-JHM ts mutants used in this study are presented in Table 1. wt MHV-JHM is highly neurotropic and induces mainly acute fatal encephalomyelitis. Surviving animals (1–25% of those infected) develop demyelinating disease (Weiner, 1973; Lampert et al., 1973). In contrast, the five ts mutants are severely attenuated in their neurovirulance. The ts 104, ts 110 and ts 115 MHV-JHM mutants did not induce any brain disease, even after inoculation of up to 10,000 plaque forming units (PFU). The ts15 mutant induced demyelination in only about 5% of infected mice. In contrast, ts8 induced demyelination in the spinal cord in 99% of the mice infected with 500 PFU, and in over 50% of the mice infected with 50 PFU. Therefore, the ts8

TABLE 1

Characterization of MHV-JHM ts mutants

| | Efficiency of plating | Infectious dose (PFU) | Neurovirulence | | Ref. |
|---|---|---|---|---|---|
| | | | Survival (%) | Disease | |
| wt MHV-JHM[a] | 0.31 to 1.0 | 0.5 | 25 | AEM[c] | f,g |
| ts104[a] | $<10^{-4}$ | 10,000 | 100 | Negative[c] | f |
| ts110[a] | $<10^{-4}$ | 10,000 | 100 | Negative[c] | f |
| ts115[a] | $<10^{-3}$ | 10,000 | 100 | Negative[c] | f |
| ts8H[b] | $4\times10^{-4}$ | 500 | 99 | Demyelination[d] | g |
| ts15H[b] | $4.5\times10^{-4}$ | 500 | 84 | Demyelination[e] | g |

[a] Efficiency of plating on 17CL-1 cells: ratio of the titre at 38.5°C to the titre at 33°C.
[b] Efficiency of plating on NCTC-1469 cells: ratio of the titre at 39.5°C to the titre at 34°C.
[c] AEM: acute encephalomyelitis; Negative: no evidence of neuropathology in brain or spinal cord by light microscopy.
[d] 99% of surviving animals exhibited demyelination.
[e] 4.8% of surviving animals exhibited demyelination.
[f] Robb et al., 1979.
[g] Haspel et al., 1978.

mutant has been extensively used as an excellent model to study demyelination.

The primary aim of these studies was to determine whether there are any differences in the viral structural proteins expressed by cells infected with wt or ts MHV-JHM mutants. The virions of MHV-JHM contains 3 structural polypeptides: the phosphorylated nucleocapsid *N*, the transmembrane *M* protein and the *S* peplomer glycoprotein (Siddell et al., 1981; Sturman and Holmes, 1983; Bond et al., 1984). Immunoprecipitation, using specific

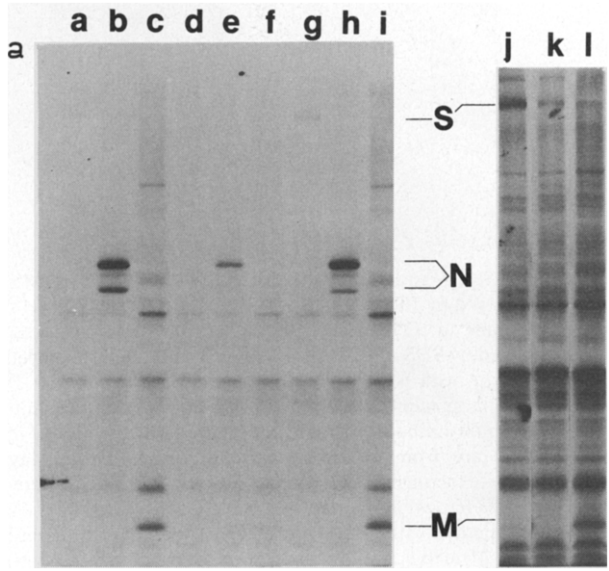


Fig. 1. See page 108 for legend.

antibodies against *S*, *M* and *N* polypeptides, of $^{35}$S-labeled lysates of L2 cells infected with either wt or ts MHV-JHM mutants, followed by SDS–PAGE analysis, revealed that all three viral proteins exhibited the same molecular masses (Fig. 1). In particular the *S* peplomer protein from cells infected with either wt or any of the five ts MHV-JHM mutants exhibited a molecular mass of 180 kDa. The *M* matrix protein had a molecular mass of 25 kDa and the *N* nucleocapsid protein of 60 kDa (Fig. 1). The remainder of our studies were concentrated on the *S* peplomer protein since it mediates many of the important biological functions of the virus. The *S* peplomer proteins expressed by L2 cells infected with either wt or any one of the 5 ts MHV-JHM mutants were compared using isoelectric focusing followed by immunoblotting.

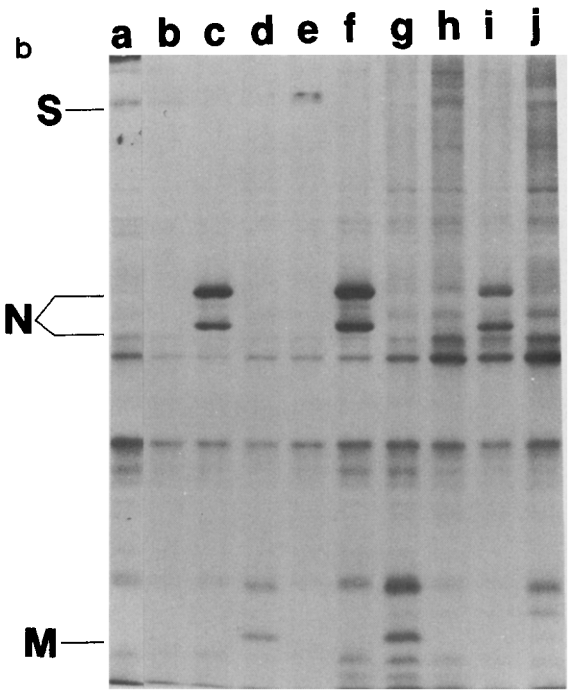


Fig. 1. Immunoprecipitation of *S,M,N* structural viral proteins of wt or MHV-JHM ts mutants or MHV-JHM by specific antibodies followed by 10% SDS-PAGE. wt and ts mutants of MHV-JHM infected cells were labeled with $^{35}$*S*-methionine at 12 h after infection and cytoplasmic extracts were prepared, immunoprecipitated and analysed by SDS–PAGE as described in 'Materials and Methods'. A. Cells were infected with ts 110 (lanes a,b,c,j), with ts 115 (lanes d,e,f,k), or with ts 104 (lanes g,h,i,l). Aliquots of infected cell extracts were immunoprecipitated with mouse anti-*S* monoclonal antibody 1.38.1. (lanes a,d,g,j,k); mouse anti-*N* monoclonal antibody 1.16.1. (lanes b,e,h); or mouse anti-*M* monoclonal antibody J.2.7.(lanes c,f,i,l). Lanes j,k,l are from an autoradiogram exposed seven days longer than the autoradiogram (from the same experiment) shown in lanes a,d,f. B. Cells were infected with ts15 mutant (lanes a,b,c,d); or with ts8 (lanes e,f,g); or with wt MHV-JHM (lanes h,i,j). Aliquots of lysates of infected cells were immunoprecipitated with anti-*S* mab 1.38.1. (lanes a,b,e,h); mouse anti-*N* mab 1.16.1. (lanes c,f,i) or with mouse anti-*M* mab J.2.7. (lanes d,g,j). Lane a is from the autoradiogram exposed seven days longer than the autoradiogram (from the same experiment) shown in lane b.

Different aliquots of L2 cells infected with either wt or any one of the 5 ts MHV-JHM mutants were subjected to isoelectric focusing as described in Materials and Methods. The focused MHV viral proteins were then transferred to NC sheets and the *S* peplomer proteins were identified using specific goat anti-*S* antibody. Bound anti-*S* antibodies were detected using horseradish peroxidase-conjugated rabbit anti-goat IgG and diaminobenzidine as substrate (Fig. 2). wt MHV-JHM *S* peplomer protein displayed microheterogeneity on isoelectric focusing and exhibited eight bands, in the range of p*I* between 6 to 7.45. The *S* peplomer protein of the ts mutants was also resolved into several bands (six or seven) with p*I* in the range of 6 to 7.45. Comparison of the staining pattern of wt MHV *S* peplomer protein with each one of the 5 ts MHV-JHM mutants revealed significant differences. Furthermore, there were significant differences in the staining pattern of the *S* proteins between all five ts MHV-JHM mutants. Control experiments where the NC sheets were reacted with normal goat serum instead of specific goat anti-*S* antibody revealed no visible bands (data not shown).

Immunoprecipitation of the *S* peplomer protein, using goat-anti *S* specific antibody, from lysates of L2 cells infected with the 5 ts MHV-JHM mutants, followed by SDS–PAGE, revealed that they exhibited identical apparent molecular weight (Fig. 1). In contrast, Taguchi and Flemming (1989) reported that six other MHV-JHM variants exhibited marked differences in the size of their *S* peplomer protein. Furthermore, viral isolates from the brains of rats

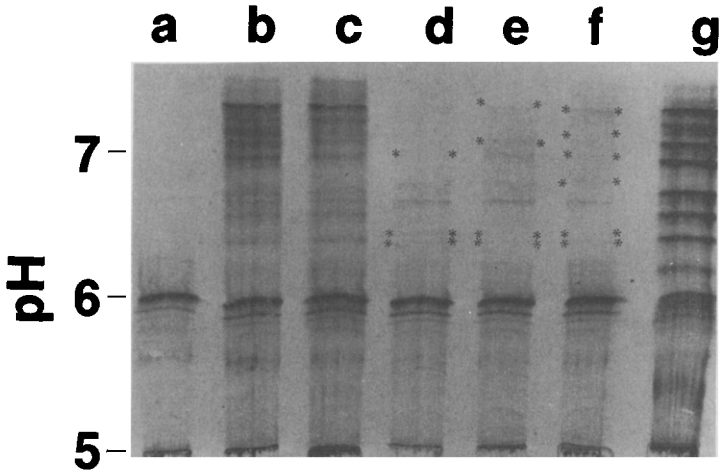


Fig. 2. Aliquots of nonlabeled lysates of cells either mock infected (lane a); or infected with ts105 (lane b); or ts110 (lane c); or ts104 (lane d); or ts8 (lane e); or ts15 (lane f) or with wt MHV-JHM (lane g) were subjected to isoelectric focusing as described in Materials and Methods. Subsequently, the separated bands were transferred to NC sheets incubated with goat anti-*S* antibody. Bound antibodies were detected using horseradish peroxidase-conjugated rabbit anti-goat IgG and visualised using diaminobenzidine as the substrate.

infected with MHV-JHM or from cultures of astrocytes persistently infected with MHV-JHM exhibited significant differences in the size of *S* peplomer protein in comparison to that of the parent virus (Taguchi et al., 1985; Morris et al., 1989).

The *S* peplomer protein of MHV is involved in cell to cell fusion, induction of neutralizing antibodies, attachment to the MHV cellular receptor and is a target of cell-mediated immune responses (Collins et al., 1982; Holmes et al., 1981; Wege et al., 1988; Wysocka et al., 1989; Williams et al., 1990). It is an important determinant of cell and organ tropism of MHV and may have a key role in the evolution of persistent infections. Recently, we have demonstrated a molecular mimicry between *S* peplomer protein and Fc receptor (Oleszak and Leibowitz 1990a, 1990b; Oleszak at al., 1992). The *S* peplomer protein plays a major role in establishing persistent infection.

Major differences were found in the microheterogeneity of the *S* peplomer proteins between the wt and all five of the ts MHV-JHM mutants. This finding is not unexpected since the *S* peplomer protein has 21 glycosylation sites (Schmidt et al., 1987). The *S* peplomer protein like many other *N*-linked glycoproteins contains both mannose and fucose, but no *N*-acetylgalactosamine (Holmes et al., 1981). It is synthesized on membrane-bound ribosomes as *N*-glycosylated polypeptide with molecular weight of 150,000. During transport through Golgi, glycosylation take place resulting in a 180,000 mol wt. *S* glycoprotein (Holmes et al., 1981; Siddell et al., 1981). The fact that the *S* peplomer protein of the ts mutants and the wt MHV-JHM have identical molecular weights suggest that the microheterogeneity that we observed is due to differences in glycosylation. It remains to be established whether these differences in glycosylation of the *S* peplomer protein plays a role in the different neuropathogenic properties of the wt and the ts MHV-JHM mutants. It is not clear at present if changes in amino acid sequence or conformation might be responsible for these changes in glycosylation.

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